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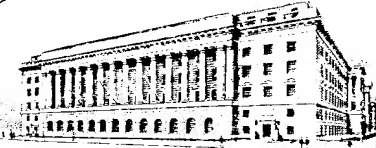
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It would be highly desirable to be provided with to the use of an acrosomal sperm protein in immunocontraception of male and female subjects.

It would be highly desirable to be provided  
5 with to the use of an acrosomal sperm protein as a marker for fertility.

**SUMMARY OF THE INVENTION**

One aim of the present invention is to provide  
10 the use of acrosomal sperm protein in immunocontraception of male and female subjects.

In accordance with the present invention there is provided a method of immunocontraception of a male or female subject, which comprises administering to  
15 said male or female subject an antigenic amount of P34 or an antigenic fragment thereof to elicit an immunocontraception response by said male or female subject.

The preferred P34 protein used has the sequence  
20 identified as SEQ ID NO:3 and the preferred antigenic fragment thereof include, without limitation, MELFLAGRRVC (SEQ ID NO:4) OR CSQDYAEPNPTWQV (SEQ ID NO:5).

An immunocontraceptive vaccine for male or  
25 female subject, which comprises an antigenic amount of P34 or an antigenic fragment thereof in association with a suitable pharmaceutically acceptable carrier, wherein said vaccine elicits an immunocontraception response by said male or female subject after its  
30 administration.

In accordance with the present invention there is provided a probe as a marker for male or female fertility, which comprises a cDNA sequence capable of hybridizing under stringent conditions with human  
35 acrosomal sperm protein P34.

In accordance with the present invention there is provided a method for the diagnosis of male or female infertility which comprises the steps of:

- a) determining the amount of human P34 in a sperm or ovule sample; and
- b) comparing the determined amount of step a) with a fertile control sample.

The amount of human P34 in step a) may be determined using an antibody raised against human P34.

In accordance with the present invention there is provided a kit for the diagnosis of male or female infertility which comprises:

- a) an anti-P34 antibody enzyme-labeled;
- b) an enzyme substrate; and
- c) a fertile control sample.

A calibration curve for the amount of human P34 may be obtained using the fertile control sample of component (c) above

For the purpose of the present invention the following terms are defined below.

The term "antigenic fragment" is intended to mean any fragment of said protein which is capable of eliciting an immune response pursuant to its administration to a subject.

#### **BRIEF DESCRIPTION OF THE DRAWINGS**

Fig. 1 illustrates the comparison of partial amino acid sequences with the corresponding amino acid sequence of P26h with AP27;

Fig. 2 illustrates Northern blot analysis of hamster total RNA from 1) testis 2) whole epididymis 3) caput epididymis 4) corpus epididymis 5) cauda epididymis 6) Fat 7) Lung 8) heart 9) liver 10) kidney 11) muscle 12) brain probed with a nP26h 710 bp cDNA

probe (upper panel) or with a positive Cyclophilin probe (lower panel);

Fig. 3 illustrates the nucleotide sequence of the P26h cDNA;

5        Fig. 4 illustrates the alignment of the deduced amino acid sequence of P26h with the AP27 and the Carbonyl Reductase;

Fig. 5 illustrates *in situ* hybridization probed with the P26h RNAs probes;

10       Fig. 6 illustrates the immunoprecipitation of P26h cDNA translational products;

Fig. 7 illustrates Northern blot analysis of human total RNA from 1) testis, 2) caput epididymidis, 4) corpus epididymidis, 5) cauda epididymidis, probed  
15       with a P34H cDNA probe; and

Fig. 8 illustrates the sequence homology of the human P34 (lower lane) counterpart of P26h (upper lane).

20       **DETAILED DESCRIPTION OF THE INVENTION**

During fertilization, mammalian spermatozoa must undergo a series of events in order to reach the oocyte surface and to perform syngamy. Some of these events occurs during the epididymal transit where  
25       spermatozoa acquire their fertilizing ability. We have previously described a hamster sperm protein, P26h, acquired during the epididymal transit. P26h shows immunocontraceptive properties when used to actively immunize male.

30       In accordance with the present invention, we have undertaken the determination of the origin and of the sequencing of the encoding cDNA of this sperm proteins showing male contraceptive properties. N-terminal sequencing of purified P26h and of peptides  
35       generated by partial proteolysis allowed partial

identification of the protein. Northern blot analysis revealed that a major transcript encoding for P26h was localized the testicular mRNA whereas no signal was detectable in other somatic tissues of the hamster. A hamster testis cDNA library was screened and a P26h encoding cDNA was cloned and sequenced. The P26h cDNA sequence revealed a 85% identity with the cDNA corresponding to mouse Adipsin and of a Carbonyl Reductase. The deduced P26h amino acid sequence possess specific domains of the Short Chain Dehydrogenase/Reductase (SDR) family proteins. Antibodies generated against synthetic peptides deduced from the cDNA sequence recognized the P26h on Western blots of detergent extracted hamster sperm proteins. On the other hand, *in vitro* translational products synthesized from the P26h cDNA was immunoprecipitated by a polyclonal antiserum produced against the purified hamster sperm P26h. *In situ* hybridization performed on tissues from the hamster reproductive tract, revealed that the P26h was principally transcribed in the seminiferous tubules and at a lower level in the corpus epididymidis. P26h shows unique feature of the SDR family that can be used to induce contraception in male.

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## **Materials and methods**

### Animals

Sexually mature Golden hamsters (*Mesocricetus auratus*; Charles River Inc., St. Constant, Qc, Canada) were used in this study. Hamsters were sacrificed under CO<sub>2</sub> atmosphere, the epididymidis were excised, defatted and dissected into caput, corpus and cauda segments. Tissues were frozen in liquid nitrogen and stored at -80°C until use. Testicular and somatic tissues were proceeded the same way. For *in situ*

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hybridization fresh tissues were rinsed in PBS-DEPC (Phosphate buffered saline-Diethyl pyrocarbonate) and fixed at 4°C for 2h in 4% (w/v) paraformaldehyde freshly prepared in PBS. Tissues were cryoprotected by sequential incubations in 10% glycerol for 1h at 4°C under agitation and then overnight in 50% OCT. Tissues were embedded in OCT and frozen in liquid nitrogen. Cryosections of ~7µm were collected on poly-L-Lysine coated slides, air-dried at -20°C, and stored at -80°C until used.

#### N-chlorosuccinimide proteolysis

Proteins from cauda epididymal spermatozoa or from the epididymal fat pad were extracted with 0.5% Nonidet™ P40 (Sigma) as previously described and submitted to preparative SDS-PAGE. After Coomassie blue staining, the bands corresponding to a MW of 26 kDa were excised, washed twice with H<sub>2</sub>O, and rinsed with a washing solution (50% (wt/vol) urea , 50% (vol/vol) ethanol). The polyacrylamide bands were incubated 30 min. in 20mg/ml N-Chlorosuccinimide in washing solution, washed in water, and then incubated 3 times for 1 hour each, in an equilibrium solution (0.0625M Tris-HCl pH 6.8, 20% (vol/vol) glycerol, 30% (vol/vol) B-mercaptoethanol, 6% (wt/vol) SDS). The band was loaded on a discontinuous polyacrylamide gel and submitted to electrophoresis. Patterns of proteins fragments were visualized by silver nitrate staining, or Western blotted using a P26h antiserum (Bérubé, B., Sullivan, R., 1994, *Biol. Reprod.*, 51: 1255-1263). Western blotted P26h fragments were also used for N-terminal sequencing as described below.

Partial amino acid sequence analysis

P26h was purified and absorbed on a piece of nitrocellulose sheet. One hundred  $\mu$ l of 50 mg/ml CNBr (Cyanogen Bromide) in 70% formic acid was added to 1 mg  
5 of the dry protein and incubated under nitrogen in the dark for 24 h. Digested peptides were loaded onto a VYDAK™ reversed-phase C18 column (250 x 1 mm) which was equilibrated with 0.1% (v/v) trifluoroacetic acid (TFA) in water and eluted with a 2-100% gradient of  
10 0.08% (v/v) TFA in 80% acetonitrile. Fractions of 0.5 ml or smaller were collected at a flow rate of 50  $\mu$ l/min. Protein sequence was performed on aliquots from one peak by automated Edman™ degradation with a pulsed-liquid phase sequencer.

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RNA extraction

Tissues were homogenized with a Polytron™ in 1.5 ml of a fresh homogenization buffer solution (4M guanidium thiocyanate, 25mM sodium citrate pH 7, 0.5% sarcosyl, 0.1M 2-mercaptoethanol). One ml of Cesium  
20 Chloride-homogenization buffer (2g of CsCl/2.5 ml) was added to the tissue lysates. This was layered on cushion solution (5.7M CsCl, 0.1M EDTA, pH 7.5) and centrifuged at 60 000g overnight. The RNA pellet was  
25 resuspended in TES solution (10mM tris-HCl, 5mM EDTA, 1% SDS, pH 7.4) and extracted with phenol/chloroform and chloroform/alcohol isoamyl 24:1. RNA was precipitated with 0.1 vol. of sodium acetate (3M, pH 5.2) and 2.5 vol. of ethanol 95%. The RNA pellet were  
30 resuspended in DEPC water. The RNA quality was evaluated by electrophoresis on a 1% agarose gel. All solutions were made with DEPC water.

#### Northern blot analysis

Total RNA (20 µg) prepared from hamster and human tissues were electrophorized on 1% agarose-formaldehyde gels and transferred to a nylon membrane (Quiagen, Santa Clarita, CA) using 20x SSC (3M NaCl, 0.3M Na-Citrate). Air dried Northern blots were UV cross-linked and prehybridized at 42°C for 4 h in 50% (vol/vol) formamide, 0.75 M NaCl, 0.05 M NaH<sub>2</sub>PO<sub>4</sub>, 0.005M EDTA, 2 X Denhardt's reagent [0.2% (wt/vol) Ficol 400, 0.2% (wt/vol) polyvinylpyrrolidone, 0.2% (wt/vol) BSA], 0.2 mg/ml herring sperm DNA (Sigma Chemicals, Mississauga, ON) and 0.1% SDS. The membrane was hybridized overnight at 42°C in the same solution, to which [ $\alpha$ -<sup>32</sup>P] dCTP-labeled DNA probes were added. The membranes were then washed twice in 0.1 x SSC-0.1% SDS followed by a third wash of 30 min. at 65°C in 0.1 x SSC-0.1% SDS, and exposed on Kodak™ X-O-Mat film with intensifying screens for 6-18 h at -80°C. A RNA ladder (1.6-7.4 kb; Boehringer Mannheim, Laval, QC) was electrophoresed in parallel and Cyclophilin probe was used as a constitutive internal control.

#### RT-PCRs production of a P26h cDNA probe

The first amino acids sequence obtained (MKLNFSXLRLVTGAGKGIG) showed high homology with the peptide sequence of the Adipsin: a marker of adipocytes differentiation. From the nucleic acid sequence of the adipsin, two primers were selected according to OLIGO 4.01™ primer analysis software (National Biosciences, Plymouth, MN), chemically synthesized (sense downstream 5'-GTG ACA GGG GCA GGG AAA GGG-3' and antisense upstream 5'-GCA ACT GAG CAG ACT AGG AGG-3') and used for RT-PCR on the total RNA from hamster's testis.

Briefly, 5 µg of the total testis RNA were incubated with 0.5 µg oligo deoxythymidine primer at

70°C for 10 min. in a final volume of 12 µl and then kept on ice. Samples were then incubated for 60 min. at 42°C in a reaction mixture containing 4 µL of 5X buffer (250 mM Tris-HCl, 375 mM KCl, 15 mM MgCl<sub>2</sub>), 10mM  
5 dithiothreitol (DDT), 1.25 mM deoxynucleotide triphosphates (dNTP) and 200 U Super Script reverse transcriptase in a final volume of 20 µl. Expression of the P26h gene was determined by amplification of the cDNA. Each reaction contained 5 µl of RT template (or  
10 water as negative control), 1.5 mM MgCl<sub>2</sub>, 1 x buffer, 0.2 mM dNTPs, 10 µM of each primer and 1.5 U Taq polymerase (Pharmacia Biotech, Baie D'Urfé, QC) in a final volume of 50 µl. The PCR cycling conditions chosen were 1 min. at 95°C, 1 min. at 60°C, 1 min. at  
15 72°C for 30 cycles, followed by a 5 min. extension at 72°C. The reaction products were analyzed electrophoresis on a 1% agarose gels; the bands were visualized by ethidium bromide staining.

The PCR band (~710 pb) was purified (Quiaquick; Quiagen), T-Cloned in pCR 3.5 (Invitrogen, San Diego, CA), and digested with EcoRI. The insert (710 bp) was separated from the vector and other fragments by electrophoresis on a 1% agarose gel, isolated from gel matrix with Na45 membrane (Schleicher & Schuell, Inc.),  
20 and random-prime labeled according to the supplier's instruction using the T7 Quick-Prime™ kit (Pharmacia Biotech, Baie D'Urfé, QC) with [ $\alpha$ -<sup>32</sup>P] dCTP. Cyclophilin cDNA was also random-prime labeled using the same procedure.

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#### Cloning and sequencing of P26h cDNA

Poly(A)<sup>+</sup>RNA from hamster and human testicular tissues was purified from total RNA solution using a poly(A)<sup>+</sup>RNA purification kit (Pharmacia Biotech, Baie  
35 D'Urfé, QC) according the supplier's instructions. The

cdna library was prepared according to the instruction  
the supplier's. Briefly, testicular poly(A)<sup>+</sup>RNA was  
reverse-transcribed and ligated into the lambda Uni-  
Zap<sup>TM</sup> XR vector (Stratagene, La Jolla, CA). The lambda  
5 library was packaged and amplified using *Escherichia*  
*coli* XL1-Blue cells, and screened with the random-prime  
labeled 710 bp P26h cDNA . The positive clones were  
isolated by plaque purification and the longest one  
(1081 bp) was subcloned into pBluscript KS+. All  
10 nucleotide sequences were determined by the  
dideoxynucleotide termination method (Sanger) using T7  
Sequenase v 2.0 kit. The labeled reaction products were  
analyzed on a DNA sequencer gel. Sequence translation  
was performed using Gene Jockey software (Biosoft,  
15 Cambridge, UK).

#### In situ hybridization

Tissues cryosections were fixed with freshly  
prepared 4% paraformaldehyde in PBS for 5 min. at RT°  
20 (Room Temperature), incubated for 10 min. in 95%  
ethanol/5% acetic acid at -20°C, and rehydrated by  
successive baths of decreasing concentrations of  
ethanol diluted with DEPC-H<sub>2</sub>O. Target RNA was unmasked  
by enzymatic digestion with 10 µg/ml proteinase K  
25 (Boehringer Mannheim) in PBS for 10 min. at 37°C,  
followed by a 5 min. incubation in 0.2% glycine.  
Sections were postfixed for 5 min. with 4%  
paraformaldehyde in PBS, acetylated with 0.25% acetic  
anhydride, 0.1 M triethanolamine, pH 8.0, for 10 min.,  
30 and finally washed with PBS.

Tissues were prehybridized for 1h with a  
preheated 250 µg/ml salmon sperm DNA in a hybridization  
solution (0.3M NaCl, 0.01M Tris-HCl pH 7.5, 1mM EDTA,  
1x Denhardt's solution, 5% dextran sulfate, 0.02% SDS  
35 and 50% formamide). Sections were then incubated

overnight at 42°C, under coverslips, with 25µl of heat-denatured antisense or sense cRNA probed with DIG (Digoxigenin: Boehringer Mannheim) according to supplier's instruction. Coverslips were removed, the  
5 sections were washed twice in 2x SSC at RT°, followed by two 10 min. washes at 42°C in 2x SSC, 1x SSC and 0.2x SSC, respectively.

Hybridization reactions were detected by immunostaining with an alkaline phosphatase-conjugated  
10 anti-DIG antibodies. Nonspecific staining was blocked by incubation for 1h with 5% (v/v) heat-inactivated sheep serum in 0.2M Tris-HCl, 0.2M NaCl, and 3% Triton™ X-100. Sections were then incubated for 2h at RT° with the alkaline phosphatase-conjugated anti-DIG  
15 antibodies diluted 1:1000 in blocking solution, washed with tris-HCl/NaCl buffer, and incubated with 0.1M tris-HCl, pH 9.5, 0.1M NaCl, and 0.01M MgCl<sub>2</sub>. The hybridization signal was visualized after a 10-15 min. incubation with the substrates nitroblue tetrazolium  
20 chloride and 5-bromo-4-chloro-3-indolylphosphate p-toluidine salt (GIBCO-BRL, Gaithersburg, MD). Levamisole (2 mM; Sigma) was added to the reaction mixture to inhibit endogenous alkaline phosphatase. Slides were immersed in 1mM EDTA, 0.01 Tris-HCl, pH  
25 7.5, washed 5 min. in H<sub>2</sub>O, counterstained with neutral red, dehydrated through baths of ethanol, cleared in xylene, and mounted with Permount (Fisher scientific, Nepean, Ontario, Canada).

30 Eukaryotic in vitro translation

*In vitro* translation was performed from circular plasmid DNA including the P26h cDNA. The TNT coupled reticulocyte lysate system was used according to the supplier's instructions (Promega, Madison, WI,  
35 USA). Briefly, 0.5 µg of circular plasmid DNA was

added directly to TNT rabbit reticulocyte lysate. T<sub>3</sub> RNA polymerase (Promega, Madison, WI, USA) and S<sup>35</sup>-methionine (10mCi/ml) were added to the translation mixture. The reaction was performed for 2 hours at 30°C. The *de novo* synthesized proteins were analyzed by SDS-PAGE according to Laemmli. The gel was soaked in an enhancer solution (Amersham), dried, and exposed on X-Omat<sup>TM</sup> AR film (Kodak) for 6 hours at room temperature. In some experiments, the translational products were submitted to NCS proteolysis (as described above) before electrophoretic analysis.

In some experiments, the translational products were immunoprecipitated using an anti-P26h antiserum. 5 µl of the translation reaction mixtures were incubated 1 h at room temperature with the P26h antiserum (previously described Bérubé, B., Sullivan, R., 1994, *Biol. Reprod.*, 51: 1255-1263) or the control serum, both diluted in Tris-saline (50mM Tris-HCl, 150mM NaCl, pH 7.5). 50µl of packed protein-A sepharose (Pharmacia) was added for 1 hour at room temperature. The immunoprecipitate was washed several times in tris-saline (50mM Tris-HCl, 500mM NaCl, pH 7.5). The immune complexes were dissociated in SDS-PAGE sample buffer (50mM Tris-HCl pH 6.3, 2% SDS (w/v) and 5% (v/v) B-mercaptoethanol) and submitted to SDS-PAGE according to Laemmli. The gel was enhanced, dried, and exposed on X-Omat<sup>TM</sup> AR film (Kodak) for 12 hours at room temperature.

### Results

When purified P26h was submitted to Edman degradation, 27 of the 29 amino acids generated were identified. A 17 kDa fragment obtained by NCS proteolysis of P26h allowed the identification of 15 of the 26 amino acids analyzed, whereas the fragment

obtained following CNBr treatment allowed the identification of 8 of 9 additional amino acids. For a total of 40 amino acids identified by Edman degradation of P26h peptides, 37 showed homology with a mouse Adipsin sequence (Fig. 1). This protein has been shown to be a differentiation growth factor of mouse adipoblasts.

In the hamster, like many mammalian species, the epididymis is surrounded by a fat pad. In the mouse, an Adipsin mRNA is abundant in the epididymal fat cushion. We were concerned by the possibility that P26h N-terminal sequences obtained may results from a contamination of sperm protein preparation by epididymal fat pad originating Adipsin. Band corresponding to 26kDa of a electrophoretic pattern of protein extracted from large amount of epididymal fat cushion was excised and submitted to N-chlorosuccisinimide proteolysis (NCS). This digestion did not generate fragment on SDS-PAGE electrophoretic pattern whereas P26h NCS digest generated a 22.4 kDa fragment. Moreover, only the P26h and its NCS digested fragment were detected by a P26h antiserum used to probe a corresponding immunoblot. The NCS digested fragment of the P26h was sequenced and the inner sequence revealed also a high homology level with the Adipsin. The intact 26 kDa protein from the fat pad protein extract was submitted to the same procedure and no N-terminal sequence was obtained.

RT-PCRs were performed with oligonucleotides derived from the cDNA sequence of the Adipsin. A 710 bp fragment was amplified from the hamster testis, cloned and sequenced. The sequence revealed that this fragment has 85% homology with the Adipsin cDNA. Using this fragment as a probe, we performed Northern blot analysis to determine in which tissues P26h

transcription occurs. Total RNA of several tissues were extracted and submitted to blot-hybridization analysis. The Northern blots showed that the total P26h messenger has 1081 bp and that it was transcribed exclusively in the testis. To confirm the presence of intact RNA in all samples, the same blot was probed with random cyclophilin DNA, and an intense signal was obtained in all samples (Fig. 2). By opposition, Northern blot analysis of mRNA prepared from human tissues revealed an abundant transcript in the epididymal tissues (Fig. 7).

A cDNA library was constructed in Lambda gtl1 from the hamster testicular mRNA and from the human epididymal tissues.  $10^5$  clones of the primary library (5 x  $10^5$  clones) were directly screened with the 710 pb cDNA probes. The first screening allowed the detection of 32 positive clones from which 11 were used for a second and third screening. The size of the inserts was determined by PCR and the longest insert (clone 2), was introduced in pBluescript SK(+/-) phagemid and sequenced. The P26h cDNA of 1081 bp has a 732 bp open reading frame, starting with a ATG at position 132 and a TAG stop codon at position 764, followed by a polyadenylation signal, and a poly A tail (Fig. 3). The sequence is numbered from the 5' end of the cDNA clone. The translation of the proposed open reading frame is shown below the nucleotide sequence and encode a peptide of 244 amino acids terminating by a amber codon.

The deduced amino acid sequence predicted a 26 kDa MW protein which is in agreement of the molecular weight of the P26h as determined by SDS-PAGE. The N-terminal sequence of P26h and of its generated peptides determined by Edman degradation (Fig. 1) were also in agreement with the amino acid sequence deduced

from the cDNA (Fig. 4). The P26h amino acid sequence was compared with the Adipsin and a carbonyl reductase, which showed a homology of 85% and 86% respectively. Adipsin and carbonyl reductase are members of the SDR (short side chain dehydrogenase/reductase) family proteins. The P26h also showed the conserved patterns of SDR, i.e. the NADH or NADPH coenzyme binding site and the active site which are respectively GlyXXXGlyXGly and TyrXXXLys (Fig. 4). The deduced amino acid sequence of the human homolog (Fig. 8, lower sequence) predict a 209 amino acid peptide sharing the SDR characteristic with the hamster P26h (Fig. 8, upper sequence).

Expression of P26h mRNA is detected in the testis, using non-radioactive *in situ* hybridization. Digoxigenin-labeled anti-sense probe revealed the expression of the P26h mRNA in the adult hamster testicular seminiferous tubules. By opposition to the Northern Blot analysis, *in situ* hybridization revealed a weaker signal along the epididymis, principally in the corpus portion. Digoxigenin-labeled sense probe was used as a control for nonspecific hybridization.

Using the TNT<sup>TM</sup> coupled reticulocyte lysate system, we performed *in vitro* translation with circular plasmid including P26h cDNA. We detected a 26 kDa signal with total translation products on SDS-PAGE. (Fig. 6A3) Total translation products were then submitted to immunoprecipitation with anti-P26h antibody, which permitted the detection of a unique signal of 26 kDa on SDS-PAGE. (Fig. 6A2). Total translation products were further submitted to NCS proteolysis. The NCS proteolysis generate a 17 kDa fragment on SDS-PAGE in agreement with the deduced amino acids sequence and the previous NCS proteolysis of purified P26h.

### Discussion:

During the epididymal transit, the mammalian spermatozoa acquire its fertilizing ability. One of the best documented physiological functions acquired by the spermatozoa during epididymal maturation is its ability to efficiently interact with the egg's zona pellucida. Our laboratory has been interested by these sperm surface modifications; mainly the addition of new surface proteins, or the post-translational modifications of preexisting sperm components, that are necessary to produce a functional male gamete. Using the hamster as a model, we have previously identified a sperm protein, P26h, which shows affinity for the homologous zona pellucida glycoproteins. P26h is abundant in the luminal fluid of the proximal region of the hamster epididymis, its concentration decreasing along the transit. Contemporarely, P26h accumulates on the spermatozoa during the epididymal maturation. P26h is exclusively located on the sperm surface covering the acrosomal cap of the mature spermatozoa; the subcellular domain involved in zona pellucida binding.

In accordance with the present invention, P26h has been purified following detergent extraction of cauda epididymal spermatozoa. This has been performed by preparative SDS-PAGE (Bérubé, B., Sullivan, R., 1994, *Biol. Reprod.*, **51**: 1255-1263) as well as by chromatographic procedures. In the latter case, a single spot in two dimensional gel electrophoresis was obtained, this single protein being recognized by the anti-P26h on corresponding Western blot. These two preparations of purified P26h, as well as proteolytic fragments, have been N-terminal sequenced by Edman degradation. All the amino acid sequences obtained showed high homology with mouse Adipsin (Fig. 1).

Adipsin has been described as a differentiation factor of adipoblasts in adipocytes. Adipsin mRNA has been shown to be present in high quantities in the mouse epididymal fat pad. In the hamster, as well as in the mouse, the majority of the epididymis is surrounded by a fat cushion. Considering that the purified P26h was obtained from spermatozoa recovered from the distal cauda epididymidis, we were concerned by the possibility that the N-terminal sequences were obtained from Adipsin liberated from adipocytes contaminating the sperm suspensions. This was conceivable if we considered that the Adipsin MW deduced from the mRNA sequence is of 27 kDa. Proteins from huge amount of epididymal fat pad were extracted and proceeded in parallel with cauda epididymal spermatozoa. Protein bands of 26-27 kDa were excised from preparative SDS-PAGE of proteins extracted from fat pad adipocytes and from cauda epididymal spermatozoa. Intact 26-27 kDa bands and proteolytic fragments generated by NCS (N-Chlorosuccinimide) digest were Western blotted and probed with the anti-P26h serum. The 26-27 kDa fat pad protein was undetectable by the anti-P26h antiserum (Fig. 2). Furthermore, the 26-27 kDa fat pad band and the P26h sperm protein were proceeded in parallel for N-terminal sequencing by Edman degradation. No signal was detectable when the fat pad protein was proceeded. From these results we can conclude that the N-terminal sequences were obtained did not result from a contamination of sperm preparation by the epididymal fat pad.

Northern blot analysis reveal a major P26h transcript in testicular tissues of the sexually mature hamster (Fig. 3). This mRNA is undetectable in the other tissues analyzed, including the fat pad and the epididymis (Fig. 3). This was unexpected since

it was previously reported that an *in vitro* translational product encoded by mRNA of the proximal region of the epididymis can be immunoprecipitated by anti-P26h antibodies. In this study, *in situ* hybridization confirm that a P26h transcript is predominant in the testis and, at a lower level, in the epididymis. In situ hybridization has been performed with digoxigenin-labelled RNA probes system using an anti-digoxigenin antibody that allows amplification of the signal and provide a more sensitive mRNA detection than the traditional Northern blot analysis. A faint labelling is detectable all along the epididymis, a much stronger signal being associated with the corpus (Fig. 5). In many species, the corpus region is known to be more active epididymal segment for protein synthesis and secretion. According to the Northern blot analysis (Fig. 2), P26h which is found at high concentration in the proximal region of the hamster epididymis probably originate from the testicular fluid as a secretion product of the seminiferous tubules, as suggested by the *in situ* localization of the transcript (Fig. 6). This protein may also be secreted by the corpus epididymidis. A dual testicular and epididymal origin has been described for other proteins interacting with the spermatozoa during epididymal maturation. Whether or not, the testicular and the epididymal P26h are identical or exist in different isoforms, as described for clusterin, remains to be determined.

The P26h being transcribed principally in testicular tissues (Fig. 2), a testicular cDNA was screened to clone the P26h cDNA. The longest transcript obtained from the library was sequenced and reveal a cDNA of 1081 bp coding for a 244 amino acids protein. The predicted MW of the translational product

is in agreement with the electrophoretic behaviour of P26h extracted from cauda epididymal spermatozoa. The P26h cDNA shows high sequence homology with Adipsin, as expected from N-terminal amino acid sequences and with a carbonyl reductase known to be expressed in pig lung (Fig. 4). The sequence homology between P26h and these two proteins is 86% and 85% respectively. The deduced amino acid sequence also shows a high homology of 87% with the Adipsin and 80% with the Carbonyl Reductase. Considering that P26h is a sperm protein involved in gamete interactions, the biological function of these two proteins was puzzling. Adipsin has been described as a differentiation factor of adipoblast in adipocytes and its expression has been shown to be inhibited by activators of protein kinase C. The carbonyl reductase is a homotetramer that catalyzes the oxidation of secondary alcohols and aldehydes. This enzyme has been shown to be expressed specifically in the lung and mainly distributed in the mitochondria. Nevertheless the high level of homology with Adipsin and a carbonyl reductase, P26h shows a complete different tissues distribution. P26h protein and its encoding mRNA are not expressed in the lung nor in the adipocyte (Figs. 2, and Bérubé, B., Sullivan, R., 1994, *Biol. Reprod.*, 51: 1255-1263). Adipsin and carbonyl reductase are known to be members of the short-chain dehydrogenase/reductase (SDR) superfamily and P26h shows some of these properties.

The Short-Chain Dehydrogenase/Reductase superfamily (SDR) is formed by a variety of different proteins that exhibit residue identities of only 15-30%. This low level of sequence identity between the members indicates an early divergence. This is reflected by the wide range of functions fulfilled by the members of this superfamily. There is three

classes of enzymes covering a wide range of EC numbers: 1, 4.2, 5.1, and 5.3, as well as members with unknown functions. Two consensus sequences are conserved in this family, the NAD(H) or NADP(H) binding domain, a N-terminal segment GlyXXXGLYXXGly, and the catalytic domain, a sequence TyrXXXLys. The P26h deduced amino acid sequence possess these consensus domains as well as the Gly 129, Ser 136 and Pro 179 which are conserved in more than 90% of the SDR family members (Fig. 4).

10 Polyclonal antibodies have been produced against P26h and used to document the function of this sperm protein during the fertilization processes in the hamster. When added to an *in vitro* fertilization medium, the antibodies anti-P26h inhibits in a dose-dependent manner, the sperm-zona pellucida interaction. Furthermore, active immunization of male hamsters against the purified P26h results in an immune response associated with reversible infertility. Using the anti-P26h antiserum, a human counterpart of P26h has also been identified and showed to be absent from sperm of men presenting with idiopathic infertility. In human, this proteins is also acquired by the spermatozoa during the epididymal transit. Taken together, these results clearly demonstrate the involvement of this sperm protein in the processes leading to fertilization. The P26h preparation that shows an immunocontraceptive properties is the same than the one used to determine N-terminal sequence by Edman degradation (Fig. 1). Furthermore, the polyclonal antiserum that allowed us to document the function of P26h in the processes of fertilization also react with the translational product encoded by the sequenced cDNA (Fig. 6). This clearly demonstrate that this SDR member is involved in mammalian sperm-egg interaction.

The mammalian spermatozoon is a highly polarized cell characterized by well defined membrane domains. Many sperm surface proteins have been proposed to play a role during the cascade of events occurring when the male gamete reach the oocyte. Different sperm proteins have been proposed as candidate involved in zona pellucida binding. Some of them shows enzymatic activity such as proacrosin, a trypsin like protease, a mannosidase, a galactosyltransferase, and P95: a hexokinase. The catalytic activity of these enzymes may not necessarily be involved in zona pellucida interaction, it is rather the substrate affinity that mediate this interaction. The biological function played by these proteins in gamete interactions is thus quite different than their enzymatic activity defined by their catalytic activity in cell metabolism. This discrepancy is reflected by their subcellular localization on the spermatozoon. To mediate zona pellucida recognition these enzymes must be localized at the sperm surface whereas they are classically known to be intracellular. This is well illustrated by the extracellular oriented sperm membrane mannosidase and galactosyltransferase, as well as by hexokinase which is at the surface of the mouse spermatozoa whereas it is known to be associated with the mitochondrial membrane. Like these potential zona pellucida ligand, P26h is localized at the hamster sperm surface, to the membrane domain covering the acrosome.

P26h belong to the SDR superfamily characterized by highly different members with a low level of identity. This reflect distant duplications and early divergence. As a consequence, SDR family represents a great diversity in enzymatic activities and functions. An interesting example of an alternative

function for an enzyme, is glyceraldehyde -3-phosphate dehydrogenase. This protein which is classically known as a glycolytic enzyme has been shown to act as a t-RNA binding protein with a function in cytoplasmic trafficking. SDR divergence is favourable for arising of new functions; involvement in gamete interactions may be one of these. Considering that P26h has been previously shown to be involved in gamete interaction and to possess immunocontraceptive properties, cloning of a homologous cDNA in human allow the identification of a human sperm protein with immunocontraceptive properties (Fig. 8). The blood-testis barrier is not present in the epididymis, allowing the neutralization of spermatozoa following immunization against an antigen involved in post-testicular maturation of the male gamete. The fact that the human sperm protein is specifically expressed in the epididymis (Fig. 7), strongly support its potential as an immunocontraceptive target.

The present invention will be more readily understood by referring to the following example which is given to illustrate the invention rather than to limit its scope.

#### EXAMPLE I

##### **Immunocontraception vaccine**

The human counterpart of the hamster cDNA P26h, encoded for an epididymal-specific protein.that is important in sperm function. It will be possible to target this protein by specific antibodies using an immunocontraceptive approach. Men will be immunized with a peptide corresponding to the epididymal protein. This peptide will be choosne with regards to its antigenic properties. An immune response against that specific peptide will occurred and no side effect is

expected since the selected peptide shows high specificity for an sperm-epididymal protein. The antibodies will reach the spermatozoa within the excurrent duct (epididymis) since the blood-testis barrier is not present at the level of the epididymis. The antibodies will neutralized the fertilizing ability of the spermatozoa has already shown with the hamster P26h and will confer an immuncontraceptive protection.

The peptide will be coupled to a carrier that will modulate the half-life of the circulating peptide. This will allowed the control on the period of contraceptive protection. The peptide-carrier will be emulsified in an adjuvant and administrated by usual immunization route.

In men under such an immunocontraceptive regiment, the circulating titer of anti-peptide antibodies will be an indication of the contraceptive efficiency. Expected reversibility will be predicted by standard immunological determination of the titer of antibodies specific to the specific peptide.

While the invention has been described in connection with specific embodiments thereof, it will be understood that it is capable of further modifications and this application is intended to cover any variations, uses, or adaptations of the invention following, in general, the principles of the invention and including such departures from the present disclosure as come within known or customary practice within the art to which the invention pertains and as may be applied to the essential features hereinbefore set forth, and as follows in the scope of the appended claims.

SEQUENCE LISTING

(1) GENERAL INFORMATION:

- (i) APPLICANT: Sullivan, Robert  
Bérubé, Bruno  
Légaré, Christine  
Gaudreault, Christian
- (ii) TITLE OF INVENTION: Acrosomal Sperm Protein And  
Uses Thereof
- (iii) NUMBER OF SEQUENCES: 5
- (iv) CORRESPONDENCE ADDRESS:
  - (A) ADDRESSEE: Swabey Ogilvy Renault
  - (B) STREET: 1600 - 1981 McGill College
  - (C) CITY: Monteval
  - (D) STATE: QC
  - (E) COUNTRY: Canada
  - (F) ZIP: H3A 2Y3
- (v) COMPUTER READABLE FORM:
  - (A) MEDIUM TYPE: Diskette
  - (B) COMPUTER: IBM Compatible
  - (C) OPERATING SYSTEM: Windows
  - (D) SOFTWARE: FastSEQ for Windows Version 2.0b
- (vi) CURRENT APPLICATION DATA:
  - (A) APPLICATION NUMBER:
  - (B) FILING DATE:
  - (C) CLASSIFICATION:
- (vii) PRIOR APPLICATION DATA:
  - (A) APPLICATION NUMBER:
  - (B) FILING DATE:
- (viii) ATTORNEY/AGENT INFORMATION:
  - (A) NAME: Murphy, Kevin P
  - (B) REGISTRATION NUMBER: 26,674
  - (C) REFERENCE/DOCKET NUMBER: 13045-2"US" FC/CC
- (ix) TELECOMMUNICATION INFORMATION:
  - (A) TELEPHONE: 514-845-7126
  - (B) TELEFAX: 514-288-8389
  - (C) TELEX:

(2) INFORMATION FOR SEQ ID NO:1:

- (i) SEQUENCE CHARACTERISTICS:
  - (A) LENGTH: 1081 base pairs
  - (B) TYPE: nucleic acid
  - (C) STRANDEDNESS: single
  - (D) TOPOLOGY: linear
- (ii) MOLECULE TYPE: cDNA

(ix) FEATURE:

- (A) NAME/KEY: Coding Sequence
- (B) LOCATION: 124...853
- (D) OTHER INFORMATION:

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:1:

GTCCCTGGAG	GTGGCTGTA	GGATTCAGGT	GGCTTGCTCA	GGCTGGGATC	AAGGACACAG	60
AGC	ATG	AAG	CTG	AAT	TTC	120
Met	Lys	Leu	Asn	Phe	Thr	168
1		5			10	15
GGG	AGA	GGG	ATT	GGG	CGA	216
Gly	Arg	Gly	Ile	Gly	Arg	20
						25
GCC	AAA	GTG	GTG	GCC	GTG	264
Ala	Lys	Val	Val	Ala	Val	35
						40
GCC	AAA	GAG	TGT	CCG	GGC	312
Ala	Lys	Glu	Cys	Pro	Gly	50
						55
TGG	GAG	GCC	ACA	GAG	AAG	360
Trp	Glu	Ala	Thr	Glu	Lys	65
						70
CTG	GTG	AAC	AAT	GCG	GCG	408
Leu	Val	Asn	Asn	Ala	Ala	80
						85
ACC	AAG	GAG	GTC	TTT	GAC	456
Thr	Lys	Glu	Val	Phe	Asp	100
						105
CTG	CAA	GTG	TCC	CAG	ATG	504
Leu	Gln	Val	Ser	Gln	Met	115
						120
GCA	GGA	TCC	ATT	GTC	AAC	552
Ala	Gly	Ser	Ile	Val	Asn	130
						135
CCT	GGT	CTG	GCC	ACG	TAC	600
Pro	Gly	Leu	Ala	Thr	Tyr	145
						150
ACC	AAA	GCC	ATG	GCC	ATG	648
Thr	Lys	Ala	Met	Ala	Met	160
						165
TCT	GTA	AAC	CCT	ACC	GTG	696
Ser	Val	Asn	Pro	Thr	Val	180
						185

GCA GAC CCG GAA TTT GCC AAG AAG CTC AAG GAG CGC CAC CCA CTG AGG	744
Ala Asp Pro Glu Phe Ala Lys Lys Leu Lys Glu Arg His Pro Leu Arg	
195 200 205	
AAG TTC GCA GAG GTG GAG GAC GTG GTC AAC AGC ATC CTC TTC CTG CTC	792
Lys Phe Ala Glu Val Glu Asp Val Val Asn Ser Ile Leu Phe Leu Leu	
210 215 220	
AGC GAC AGC AGC GCC TCT ACC AGC GGC TCT GGC ATC CTG GTG GAC GCT	840
Ser Asp Ser Ser Ala Ser Thr Ser Gly Ser Gly Ile Leu Val Asp Ala	
225 230 235	
GGT TAC CTG GCC T CCTAGACGGC CCAGGTGCAG GGGACTCCTG GAGACTTCCC	893
Gly Tyr Leu Ala Ser	
240	
TGGCCTCACC CTTACATCAA GACCCCGCCT TCAACCCAAC CCAATAATTT TGTTTGAATC	953
CTGTAGAGCC CCACCCACAC CACATCCATC CCAACTTTA GACTCCGGGA TCCCGCCATT	1013
CCATACCAGC TATGCTGAGA TAATTTGATT AAATAAGTAT CCCAAACCAC AAAAAAAAAA	1073
AAAAAAAA	1081

(2) INFORMATION FOR SEQ ID NO:2:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 244 amino acids
- (B) TYPE: amino acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(v) FRAGMENT TYPE: internal

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:2:

Met	Lys	Leu	Asn	Phe	Thr	Gly	Leu	Arg	Ala	Leu	Val	Thr	Gly	Ala	Gly
1				5					10					15	
Arg	Gly	Ile	Gly	Arg	Gly	Thr	Ala	Lys	Ala	Leu	His	Ala	Ser	Gly	Ala
			20					25					30		
Lys	Val	Val	Ala	Val	Ser	Leu	Ile	Asn	Glu	Asp	Leu	Val	Ser	Leu	Ala
			35				40					45			
Lys	Glu	Cys	Pro	Gly	Ile	Glu	Pro	Val	Cys	Val	Asp	Leu	Gly	Asp	Trp
	50					55				60					
Glu	Ala	Thr	Glu	Lys	Ala	Leu	Gly	Arg	Ile	Gly	Pro	Val	Asp	Leu	Leu
65					70					75				80	
Val	Asn	Asn	Ala	Ala	Val	Ala	Leu	Val	Gln	Pro	Phe	Ile	Gln	Ser	Thr
			85						90					95	
Lys	Glu	Val	Phe	Asp	Arg	Ser	Phe	Asn	Val	Asn	Val	Arg	Ser	Val	Leu
			100					105					110		
Gln	Val	Ser	Gln	Met	Val	Ala	Lys	Gly	Met	Ile	Asn	Arg	Gly	Val	Ala
			115				120					125			
Gly	Ser	Ile	Val	Asn	Ile	Ser	Ser	Met	Val	Ala	Tyr	Val	Thr	Phe	Pro
			130			135					140				
Gly	Leu	Ala	Thr	Tyr	Ser	Ser	Thr	Lys	Gly	Ala	Ile	Thr	Met	Leu	Thr
145					150					155				160	
Lys	Ala	Met	Ala	Met	Glu	Leu	Gly	Pro	Tyr	Lys	Ile	Arg	Val	Asn	Ser
				165				170						175	
Val	Asn	Pro	Thr	Val	Val	Leu	Thr	Asp	Met	Gly	Lys	Lys	Val	Ser	Ala
			180					185					190		

Asp Pro Glu Phe Ala Lys Lys Leu Lys Glu Arg His Pro Leu Arg Lys  
 195 200 205  
 Phe Ala Glu Val Glu Asp Val Val Asn Ser Ile Leu Phe Leu Leu Ser  
 210 215 220  
 Asp Ser Ser Ala Ser Thr Ser Gly Ser Gly Ile Leu Val Asp Ala Gly  
 225 230 235 240  
 Tyr Leu Ala Ser

(2) INFORMATION FOR SEQ ID NO:3:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 912 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(ix) FEATURE:

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:3:

GACAAAGCT	GGAGTCCAC	CGCGGTGGCG	GCCGCTCTAG	AACTAGTGA	TCCCCGGGC	60
TGCAGGAATT	CGGCACGAGC	CGACATGGAG	CTGTTCTCG	CGGGCCGCCG	GGTGCTGGTC	120
ACCGGGGCAG	GCAAAGGTAT	AGGGCGCGGC	ACGGTCCAGG	CGCTGCACGC	GACGGGCGCG	180
CGGGTGGTGG	CTGTGAGCCG	GACTCAGGCG	GATCTTGACA	GCCTTGTCGG	CGAGTGCCCG	240
GGGATAGAAC	CCGTGTGCGT	GGACCTGGGT	GACTGGGAGG	CCACCGAGCG	GGCGCTGGGC	300
AGCGTGGGCC	CCGTGGACCT	GCTGGTGAAC	AACGCCGCTG	TCGCCCTGCT	GCAGCCCTTC	360
CTGGAGGTCA	CCAAGGAGGC	CTTTGACAGA	TCCTTGAGG	TGAACCTGCG	TGCGGTCATC	420
CAGGTGTCGC	AGATTGTGGC	CAGGGGCTTA	ATAGCCCGGG	GAGTACCAGG	GGCCATCGTG	480
AATGTCTCCA	GCCAGTGCTC	CCAGCGGGCA	GTAACCTAAC	ATAGCGTCTA	CTGCTCCACC	540
AAGGGTGCCC	TGGACATGCT	GACCAAGGTG	ATGCCCTTAG	AGCTCGGGCC	CCACAAGATC	600
CGAGTGAATG	CAGTAAACCC	CACAGTGGTG	ATGACGTCCA	TGGCCAGCCC	ACCTGGAGTG	660
ACCCCCACAA	GCCAAGACTA	TGCTGAACCG	AATCCCACTT	GGCAAGTTTG	CTGAGGTAGA	720
GCACGTGGTG	AACGCCATCC	TCTTTCTGCT	GAGTGACCGA	AGTGGCATGA	CCACGGGTTC	780
CACTTTGCCG	GTGGAAGGGG	GCTTCTGGGC	CTGCTGAGCT	CCCTCCACAC	ACCTCAAGCC	840
CCATGCCGTG	CTCATCCTAC	CCCCAATCCC	TCCAATAAAC	CTGATTCTGC	TCCCCAAAAA	900
AAAAAAAAAA	AA					912

(2) INFORMATION FOR SEQ ID NO:4:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 11 amino acids
- (B) TYPE: amino acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:4:

Met Glu Leu Phe Leu Ala Gly Arg Arg Val Cys  
 1 5 10

(2) INFORMATION FOR SEQ ID NO:5:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 14 amino acids

(B) TYPE: amino acid  
(C) STRANDEDNESS: single  
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:5:

Cys Ser Gln Asp Tyr Ala Glu Pro Asn Pro Thr Trp Gln Val  
1 5 10

WHAT IS CLAIMED IS:

1. A method of immunocontraception of a male or female subject, which comprises administering to said male or female subject an antigenic amount of P34 or an antigenic fragment thereof to elicit an immunocontraception response by said male or female subject.

2. The method of claim 1, wherein said P34 protein has the sequence set forth in Seq ID NO:3 and the preferred antigenic fragment is selected from the group consisting of MELFLAGRRVC (SEQ ID NO:4) and CSQDYAEPNPTWQV (SEQ ID NO:5).

3. An immunocontraceptive vaccine for male or female subject, which comprises an antigenic amount of P34 or an antigenic fragment thereof in association with a suitable pharmaceutically acceptable carrier, wherein said vaccine elicits an immunocontraception response by said male or female subject after its administration.

4. A probe as a marker for male or female fertility, which comprises a cDNA sequence capable of hybridizing under stringent conditions with human acrosomal sperm protein P34.

5. A method for the diagnosis of male or female infertility which comprises the steps of:

- a) determining the amount of human P34 in a sperm or ovule sample; and
- b) comparing the determined amount of step a) with a fertile control sample.

7/ A kit for the diagnosis of male or female infertility which comprises:

8. The kit of claim 7, which further comprises a calibration curve for the amount of human P34 may be obtained using the fertile control sample of component (c) above.

ABSTRACT OF THE INVENTION

The present invention relates to the use of acrosomal sperm protein in immunocontraception of male and female subjects and uses thereof as a marker for fertility.

20250610 14:05:00

# Combined Declaration for Patent Application and Power of Attorney

As a person named inventor, I hereby declare that:

My residence, post office address and citizenship are as stated below next to my name.

I believe that I am the original, first and sole inventor (if only one name listed below) or an original, first and joint inventor (if plural names are listed below) of the subject matter which is claimed and for which a patent is sought on the invention entitled ACROSOMAL SPERM PROTEIN AND USES THEREOF, the specification of which

☒ is attached hereto.

☐ was filed on \_\_\_\_\_ as Application No. \_\_\_\_\_  
and (if applicable) was amended on \_\_\_\_\_

I hereby state that I have reviewed and understand the contents of the above-identified specification, including the claims, as amended by any amendment referred to above.

I acknowledge the duty to disclose information which is material to the examination of this application in accordance with Title 37, Code of Federal Regulations, § 1.56(a).

I hereby claim foreign priority benefits under Title 35, United States Code § 119 of any foreign application(s) for patent or inventor's certificate listed below and have also identified below any foreign application for patent or inventor's certificate having filing date before that of the application on which priority is claimed:

## Prior Foreign Application(s)

Number	Country	Day/Month/Year Filed	Priority Claimed

I hereby claim the benefit under Title 35, United States Code, § 120 of any United States Application(s) listed below and, insofar as the subject matter of each of the claims of this application is not disclosed in the prior United States application in the manner provided by the first paragraph of Title 35, United States Code, § 112, I acknowledge the duty to disclose material information as defined in Title 37, Code of Federal Regulations, § 1.56(a) which occurred between the filing date of the prior application and the national or PCT international filing date of this application:

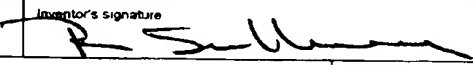
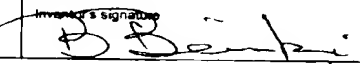
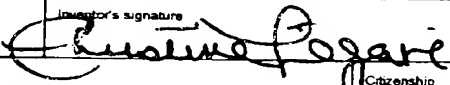

Application Serial No.	Day/Month/Year Filed	Status (Patented, Pending, Abandoned)

I hereby appoint the following attorneys, with full power of substitution, association, and revocation, to prosecute this application and to transact all business in the Patent and Trademark Office connected therewith.

ROBERT MITCHELL, Registration No. 25,007; GUY HOULE, Registration No. 24, 971; PAUL MARCOUX, Registration No. 24,990; KEVIN P. MURPHY, Registration No. 26,674; ROBERT CARRIER, Registration No. 30,726; MICHEL J. SOFIA; Registration No. 37,017; and FRANCE CÔTÉ, Registration No. 37,037; JAMES ANGLEHART, Reg. No. 38,796, and address all correspondence to:

**SWABEY OGILVY RENAULT**  
1981 McGill College Ave., Suite 1600  
Montreal, Quebec, Canada  
H3A 2Y3  
Telephone No. (514) 845-7126

I hereby further declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code and that such willful false statements may jeopardize the validity of the application or any patent issued thereon.

Full name of sole or first inventor Robert Sullivan	Inventor's signature 	Date 4/6/98
Residence and Post Office address 3261 Chemin St-Louis, Ste-Foy, Québec, Canada G1W 1S1	Citizenship Canadian	
Full name of second inventor Bruno Bérubé	Inventor's signature 	Date 4-6-98
Residence and Post Office address 76 du Buisson, Loretteville, Québec, Canada G2A 1M7	Citizenship Canadian	
Full name of sole or first inventor Christine Légaré	Inventor's signature 	Date 4-6-98
Residence and Post Office address 294 Paquet, St-Rédempteur, Québec, Canada G6K 1M3	Citizenship Canadian	
Full name of second inventor Christian Gaudreault	Inventor's signature 	Date 4-6-98
Residence and Post Office address 710 Horizon Apt. 7, Ste-Foy, Québec, Canada G1V 2X7	Citizenship Canadian	

Applicant or Patentee: Robert Sullivan, et al  
Serial or Patent No.: \_\_\_\_\_ Atty. Dkt. No.: 13045-2"US" FC/CC/LM  
Filed or Issued: \_\_\_\_\_  
For: ACROSOMAL SPERM PROTEIN AND USES THEREOF

**VERIFIED STATEMENT (DECLARATION) CLAIMING SMALL ENTITY STATUS**  
**[37 CFR 1.9(f) AND 1.27 (c)] - SMALL BUSINESS CONCERN**

I hereby declare that I am

- ☐ ( ) the owner of the small business concern identified below;  
☒ (X) an official of the small business concern empowered to act on behalf of the concern identified below:

NAME OF CONCERN IMMUCON INC.  
ADDRESS OF CONCERN 1224 Stanley Street, Suite 311, Montreal, Quebec, Canada H3B 2S7

I hereby declare that the above identified small business concern qualifies as a small business concern as defined in 13 CFR 121.3-18, and reproduced in 37 CFR 1.9(d), for purposes of paying reduced fees under section 41(a) and (b) of Title 35, United States Code, in that the number of employees of the concern, including those of its affiliates, does not exceed 500 persons. For purposes of this statement, (1) the number of employees of the business concern is the average over the previous fiscal year of the concern of the persons employed on a full-time, part-time or temporary basis during each of the pay periods of the fiscal year, and (2) concerns are affiliates of each other when either, directly or indirectly, one concern controls or has the power to control the other, or a third party or parties controls or has the power to control both.

I hereby declare that rights under contract or law have been conveyed to and remain with the small business concern identified above with regard to the invention, entitled ACROSOMAL SPERM PROTEIN AND USES THEREOF by inventor(s) Robert Sullivan, Bruno Bérubé, Christine Légaré and Christian Gaudreault described in:

- ☒ (X) the specification filed herewith;  
☐ ( ) application serial no. \_\_\_\_\_, filed \_\_\_\_\_;  
☐ ( ) patent no. \_\_\_\_\_, issued \_\_\_\_\_.

If the rights held by the above identified small business concern are not exclusive, each individual, concern or organization having rights to the invention is listed below\* and no rights to the invention are held by any person, other than the inventor who could not qualify as an independent inventor under 37 CFR 1.9(c) if that person made the invention, or by any concern which would not qualify as a small business concern under 37 CFR 1.9(d) or a nonprofit organization under 37 CFR 1.9(e). \*NOTE: Separate verified statements are required from each named person, concern or organization having rights to the invention averring to their status as small entities. [37 CFR 1.27]

NAME \_\_\_\_\_  
ADDRESS \_\_\_\_\_  
☐ ( ) INDIVIDUAL ☐ ( ) SMALL BUSINESS CONCERN ☐ ( ) NONPROFIT ORGANIZATION

I acknowledge the duty to file, in this application or patent, notification of any change in status resulting in loss of entitlement to small entity status prior to paying, or at the time of paying, the earliest of the issue fee or any maintenance fee due after the date on which status as a small entity is no longer appropriate. [37 CFR 1.28(b)]

I hereby declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true, and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under section 1001 of Title 18 of the United States Code, and that such willful false statements may jeopardize the validity of the application, any patent issuing thereon, or any patent to which this verified statement is directed.

NAME OF PERSON SIGNING Alain Bossé  
TITLE OF PERSON OTHER THAN OWNER President  
ADDRESS OF PERSON SIGNING 1224 Stanley Street, Suite 111, Montreal, Quebec,  
Canada H3B 2S7

SIGNATURE Alain Bossé DATE June 2<sup>nd</sup> 98

2009-01-01

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2009-01-01

2009-01-01

1	2	3	4	5	6	7	8	9	10	11	12
---	---	---	---	---	---	---	---	---	----	----	----

1

Fig. 2

GTCCCTGGAGGTTGGCTGTAGGATTTCAGGTGGCTTGCTCAGGCTGGG	47
ATCAAGGACACAGTGAGCAGATCAACCTTAACCTCAGCCCCCTCCC	92
CTCGCCACAGGAGGACACTGGTGTGTCAGCAGC ATG AAG CTG AAT	135
M K L N	4
TTC ACT GGT CTC AGG GCT CTG GTG ACC GGG GCA GGG AGA GGG	177
F T G L R A L V T G A G R G	18
ATT GGG CGA GGC ACT GCG AAA GCC CTG CAT GCC TCA GGA GCC	219
I G R G T A K A L H A S G A	32
AAA GTG GTG GCC GTG TCA CTC ATC AAC GAA GAC CTG GTC AGC	261
K V V A V S L I N E D L V S	46
CTG GCC AAA GAG TGT CCG GGC ATA GAG CCT GTG TGT GTG GAC	303
L A K E C P G I E P V C V D	60
CTG GGT GAC TGG GAG GCC ACA GAG AAG GCA CTG GGC CGT ATT	345
L G D W E A T E K A L G R I	74
GGC CCC GTG GAC CTG CTG GTG AAC AAT GCG GCG GTG GCG CTA	387
G P V D L L V N N A A V A L	88
GTG CAG CCT TTC ATA CAG TCT ACC AAG GAG GTC TTT GAC AGG	429
V Q P F I Q S T K E V F D R	102
TCC TTC AAT GTG AAT GTG CGC TCT GTG CTG CAA GTG TCC CAG	471
S F N V N V R S V L Q V S Q	116
ATG GTA GCC AAG GGC ATG ATT AAC CGT GGA GTG GCA GGA TCC	513
M V A K G M I N R G V A G S	130
ATT GTC AAC ATC TCC AGC ATG GTG GCC TAT GTC ACC TTC CCT	555
I V N I S S M V A Y V T F P	144
GGT CTG GCC ACG TAC AGC TCC ACC AAG GGT GCT ATA ACC ATG	597
G L A T Y S S T K G A I T M	158
CTG ACC AAA GCC ATG GCC ATG GAG CTG GGA CCA TAC AAG ATC	639
L T K A M A M E L G P Y K I	172
CGG GTG AAC TCT GTA AAC CCT ACC GTG GTG CTG ACT GAC ATG	681
R V N S V N P T V V L T D M	186
GGC AAG AAA GTC TCT GCA GAC CCG GAA TTT GCC AAG AAG CTC	723
G K K V S A D P E F A K K L	200
AAG GAG CGC CAC CCA CTG AGG AAG TTC GCA GAG GTG GAG GAC	765
K E R H P L R K F A E V E D	214

Fig. 3A

GTG GTC AAC AGC ATC CTC TTC CTG CTC AGC GAC AGC AGC GCC	807
V V N S I L F L L S D S S A	228
TCT ACC AGC GGC TCT GGC ATC CTG GTG GAC GCT GGT TAC CTG	849
S T S G S G I L V D A G Y L	242
GCC TCC TAG ACGGCCAGGTGCAGGGGACTCCTGGAGACTTCC	892
A S Amber	244
CTGGCCTCACCCCTTACATCAAGACCCCGCCTTCAACCCAACCCAATAAT	941
TITGTTCTGAATCCTGTAGAGCCCCACCCACACACATCCATCCCCAACT	990
TTAGACTCCGGGATCCCGCCATTCCATACCAGCTATGCTGAGATAATT	1038
TGATTAAATAAGTATCCCAAACCACAAAAAAAAAAAAAAAAAAAAA	1081

Fig. 3B

Fig. 4

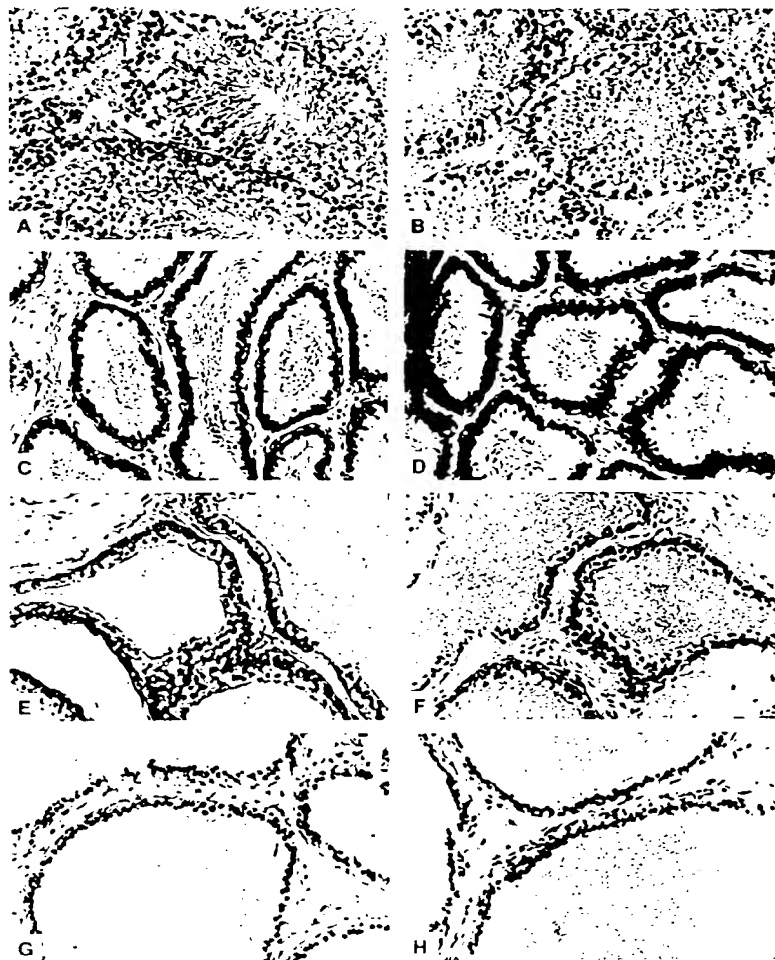


Fig. 5

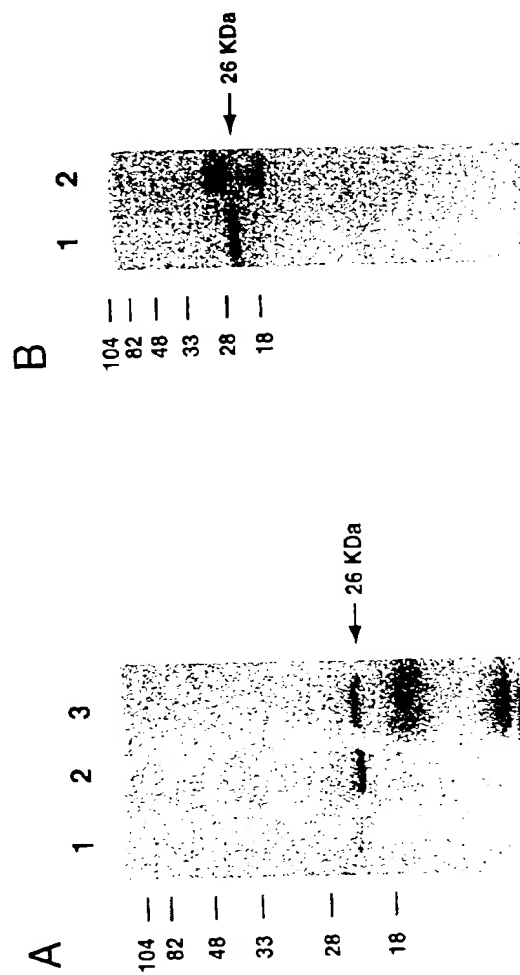


Fig. 6

		Epididymis		
		<hr/>		
M	Testis	Caput	Corpus	Cauda



← 913bp

Fig. 7

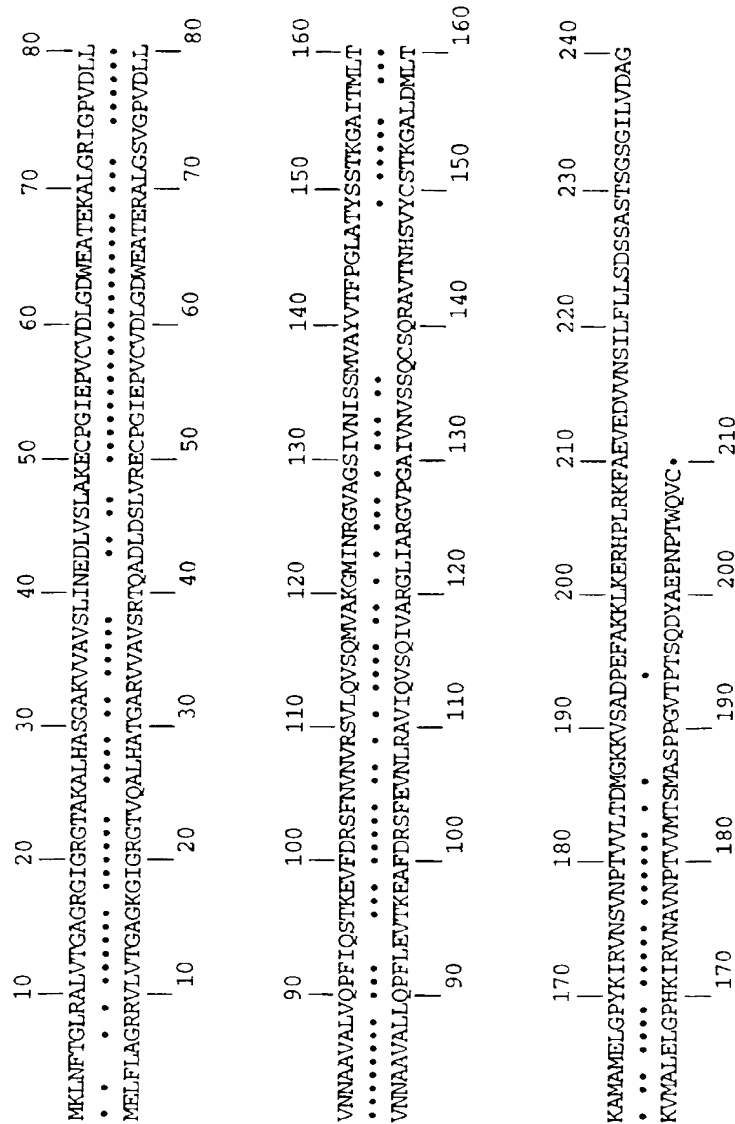


Fig. 8

